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(71) Applicant: SOMATIX THERAPY CORPORATION [US/US]; Suite 100, 950 Marina Village Parkway, Alameda, CA 94501 (US).

(72) Inventors: SHANKARA, Srinivas; Apartment E, 2255 San Jose Avenue, Alameda, CA 94501 (US). DWARKI, Varavani; Apartment N, 1175 Broadway Street, Alameda, CA 94501 (US). NIJJAR, Tarlochan; 946 Foxfire Drive, Manteca, CA 95336 (US).

(74) Agents: HALLUIN, Albert, P. et al.; Pennie & Edmonds, 1155 Avenue of the Americas, New York, NY 10036 (US).

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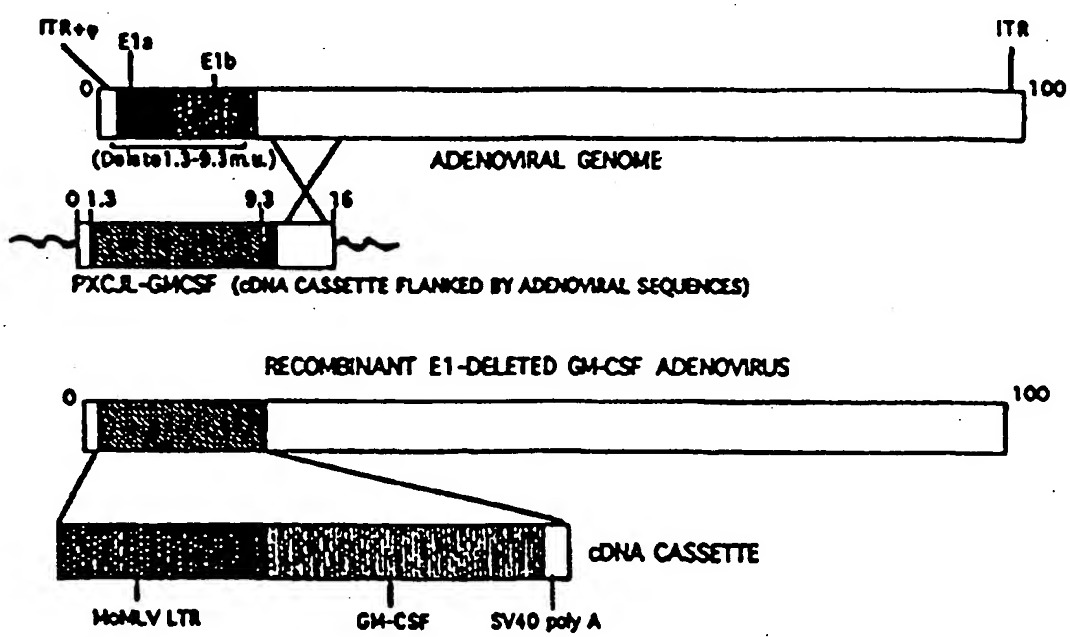

(57) Abstract

Chimeric adenovirus capable of transducing mammalian cells with DNA of interest are disclosed. The chimeric adenovirus are useful for the delivery of cloned genes into an individual and are therefore also useful for treating mammalian genetic diseases and disorders.

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CHIMERIC ADENOVIRUS FOR GENE DELIVERY

1. FIELD OF THE INVENTION

The present invention is directed to novel adenovirus vectors useful for the delivery of cloned genetic material to target cells. The chimeric adenovirus vectors comprise genetic material of interest which is flanked by adenoviral sequences, and may optionally comprise a suitable eucaryotic promoter to facilitate the expression of the genetic material of interest. The chimeric adenovirus are produced by a process involving a recombinant adenovirus vector which is used in conjunction with replication deficient helper adenovirus genomes to generate recombinantly produced chimeric adenovirus particles comprising the genetic material of interest. The resulting chimeric adenovirus may be used to infect target cells which subsequently express the cloned genetic material. One class of novel chimeric adenovirus does not contain a selectable marker which obviates the need for a selection step after the genetic material of interest has been introduced into the target cells.

2. BACKGROUND OF THE INVENTION

Mammalian cells may be transduced by any of a variety of well known processes. Techniques such as calcium phosphate precipitation and DEAE-dextran mediated transfection are widely used in the art. More recently, other techniques for delivery of exogenous DNA into cells such as electroporation or the use of liposomes have gained increased acceptance. Perhaps the most elegant methods of introducing recombinant nucleic acid into cells is viral mediated cell transduction.

Recombinant retroviruses have been widely used in gene transfer experiments (see generally, Mulligan, R.C., Chapter 8, In: Experimental Manipulation of Gene Expression, Academic Press, pp. 155-173 (1983); Coffin, J., In: RNA Tumor Viruses, Weiss, R. et al. (eds.), Cold Spring Harbor Laboratory, Vol. 2, pp. 36-38 (1985). Other eucaryotic viruses which have been

used as vectors to transduce mammalian cells include adenovirus, papilloma virus, herpes virus, adeno-associated virus, rabies virus, and the like (See generally, Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, Vol. 3:16.1-16.89 (1989).

Adenovirus have proved to be of particular interest because of several features of adenoviral biology (See generally, Berkner, K.L. (1992) Curr. Top. Microbiol. Immunol. 158:39-66). For instance, viral concentration, or titer, may be an important factor in achieving high efficiency transduction of mammalian cells. Adenovirus, by virtue of their life-style, generally allow growth conditions which result in production of higher titer stocks then other mammalian virus.

Also unlike other viruses, adenovirus capsids are not enveloped. Because of this fact, adenovirus particles are quite stable, and may retain infectivity after any of a variety of laboratory procedures. Procedures of particular interest include methods of concentrating infective virus, e.g., CsCl centrifugation, or methods that allow virus to be stored for relatively long periods while retaining substantial infectivity.

Furthermore, the expression of genes encoded by recombinant adenovirus does not require target cell proliferation or viral integration, although a small subset of the adenovirus presumably integrate into the host genome during infection. Hence, adenoviral vectors are generally better suited than other viral vectors for the transduction of postmitotic, slowly proliferating, or nonreplicating cells.

Additionally, particularly where species-specific infection is preferred, replication deficient human, or murine, adenovirus are available for the construction of recombinant virus particles that express a gene of interest. Thus, unlike transduction systems using other eucaryotic virus vectors, recombinant adenovirus can be engineered to utilize viral coat proteins which normally facilitate the normal infection of human cells or cells of other species, rather

then rely on the viral coats of a less specific, or amphotropic, nature. This species specificity appears to result in more efficient infection kinetics than can generally be obtained by virus with less specific infectivity.

An additional advantage of using adenovirus for gene delivery is that the genetic material transduced (to be expressed) into the host cell is DNA. Thus, expression of the transduced gene does not need to be preceded by reverse transcription. This is particularly advantageous where the intended recipient is undergoing treatment for the suppression of retroviral disease (i.e., AZT treatment to inhibit reverse transcriptase activity), such as treatment for acquired immunodeficiency syndrome (AIDS).

Recombinant adenoviral vectors have been generated which express a variety of genes. Perhaps most notable is the replication deficient adenovirus vector Ad.RSV that expresses incorporated genetic material of interest using an incorporated promoter from the Rous Sarcoma Virus. In particular, Ad.RSV beta gal (which expresses the bacterial β-galactosidase gene) has been used as a marker for in vivo gene transfer experiments involving salivary glands (Mastrangeli et al. (1994) Am. J. Physiol. 266:1146-1155); mesothelial cells (Setoguchi et al. (1994) Am. J. Respir. Cell. Mol. Biol. 10(4):369-377); and tumor cells (Brody et al. (1994) Hum. Gene Ther. 5(4):437-447, Chen et al. (1994) Proc. Natl. Acad. Sci., U.S.A. 91(8):3054-3057).

An ideal replication deficient adenovirus for the delivery of genetic material of interest would comprise a variety of structural and functional elements. It would readily infect target cells of interest; it would place the gene of interest under the control of a well-characterized eucaryotic promoter element; it would create a gene structure flanking the gene of interest which would provide properly spaced and oriented genetic elements to allow optimum translational efficiency and mRNA stability; and it would produce high titer and substantially helper-free stocks of the recombinant adenovirus.

3. SUMMARY OF THE INVENTION

The present invention relates to replication deficient chimeric adenovirus that allow for the rapid insertion and expression of deoxyribonucleic acid (DNA) of interest into mammalian cells, either *in vitro* or *in vivo*. The DNA of interest can optionally comprise a gene, or fraction thereof, oriented to express either a polypeptide or protein of interest, or a "sense" or "antisense" nucleic acid of structural or regulatory importance. Preferably, the DNA of interest will be placed in an expression cassette that contains a eucaryotic promoter and/or enhancer region; nucleotide sequence corresponding to a retroviral Psi-packaging site; and a substantially noncoding 3' DNA which facilitates the stability, polyadenlyation, or splicing of the transcript.

The chimeric adenovirus are thus useful for both the transduction of mammalian cells, and the expression of DNA of interest to produce regulatory factors or proteins. The regulatory factors or proteins may optionally be produced in culture or otherwise such that they can be subsequently purified and used for therapeutic, medicinal or diagnostic purposes.

The chimeric adenovirus are particularly useful for gene therapy, replacement, or insertion because of the high infectivity inherent in adenovirus biology; the high viral concentrations which may be produced during the culture and subsequent concentration of the chimeric adenovirus; and the relatively long storage life of the chimeric particles.

Either murine, or human adenovirus of serotypes A, B, or C may be used in the present invention. Of particular interest are type C adenovirus (used in the present invention) which retain infectivity while generally being considered nononcogenic.

4. BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic representation of the method of producing chimeric adenovirus via the recombination of

cotransfected plasmids. One plasmid, pXCJL-GMCSF, contains a "cassette" comprising the gene encoding the cytokine granulocyte/macrophage colony stimulating factor (GMCSF) situated such that it is transcribed, processed, and translated under the regulatory control of flanking viral sequences. The second plasmid, pJM17, comprises a replication and packaging deficient adenovirus "helper" genome. The two plasmids must recombine to produce a packagable genome, and thus substantially all of the resulting virus comprise the chimeric adenovirus desired (Recombinant E1-deleted GM-CSF adenovirus).

Figure 2 presents a schematic diagram and partial restriction map of pJM17.

Figures 3A-E disclose the DNA sequence of pXJCL-hGM-CSF (SEQ. I.D. NO. 1), the plasmid used to construct the human GM-CSF expression cassette, and in the recombinatory insertion of the GM-CSF expression cassette into the replication deficient genome contained in pJM17. The sequence of the murine GM-CSF is disclosed in foreign patent EP177568B1, herein incorporated by reference.

Figures 4A and 4B show the transient expression of human GM-CSF after one month old Balb/c mice were intramuscularly injected with either 10^9 or 10^8 pfu of Ad.hGM-CSF respectively. Serum samples were taken up to twenty one days after infection and GM-CSF levels were assayed by ELISA. Individual mice are represented by number and correspond to the indicated bars on the graphs.

Figure 5 shows the expression of human GM-CSF (as quantified by ELISA) after Ad.hGM-CSF injection and reinjection into adult Balb/C mice. Four month old Balb/C mice were injected with 10^8 pfu of Ad.hGM-CSF either I.V. (mice 103 and 105) or I.M. (mice 201, 203, and 205). All mice were reinjected (I.M.) with 10^9 pfu of Ad.hGM-CSF at day 31.

Figure 6 shows the expression of human GM-CSF (as quantified by ELISA) after Ad.hGM-CSF injection and reinjection into adult SCID mice. SCID mice were injected (I.V.) with 10^8 pfu of Ad.hGM-CSF, and GM-CSF blood serum levels were subsequently monitored. All mice were reinjected (I.M.) with 10^9 pfu of Ad.hGM-CSF at day 31, and monitored for GM-CSF expression through day 71.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention provides for chimeric adenovirus which are useful for transducing mammalian cells with DNA of interest, as well as methods of producing and using the chimeric adenovirus. Previous recombinant adenovirus expression vectors have specifically taught the expression of the genetic material of interest under the control of endogenous adenoviral promoters, or have suggested that the DNA of interest be inserted into recombinant adenovirus under the control of an RSV promoter already present in the vector Ad.RSV.

In the present system, the particular DNA of interest is first constructed as an expression cassette which comprises a gene, or portion thereof, of interest that is flanked by sequences of viral origin which are spatially organized to optimize the expression of the DNA of interest. As used herein, the term "expression" refers to the transcription of the DNA of interest, and the splicing, processing, stability, and, optionally, translation of the corresponding mRNA transcript. The recombinant DNA cassette is subsequently recombined into a replication deficient helper adenovirus to produce the infective chimeric adenovirus of interest. This method best ensures the maximal expression of the DNA of interest and additionally provides a method that is generally applicable to the relatively facile production of chimeric adenovirus which express a wide variety of DNAs.

The particular advantage of using an expression cassette stems from the fact that the recombinant Ad.RSV vector is rather large (over 36kb). This large size makes plasmids

which contain the Ad.RSV genome somewhat difficult to engineer as the number of unique (and hence useful) restriction sites tends to diminish as the amount of DNA sequence increases. Thus, the utilization of a smaller plasmid to construct the expression cassette better enables a wide variety of genetic engineering techniques which may allow the fine tuning of the expression of the DNA of interest (see generally, Sambrook et al. (1989) Molecular Cloning Vols. I-III, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, and Current Protocols in Molecular Biology (1989) John Wiley & Sons, all Vols. and periodic updates thereof, herein incorporated by reference). For instance, after the DNA of interest is placed between the desired regulatory elements (i.e., promoter and poly-adenylation signal), unwanted regions of extraneous DNA may be looped-out and deleted by site-directed mutagenesis (Krogstad and Champoux (1990) J. Virol. 64(6):2796-2801, herein incorporated by reference) such that the DNA of interest is precisely placed relative to the promoter and splicing elements, and, if a protein or polypeptide is desired, a strong Kozak translation start site (Kozak (1989) J. Cell Biol. 108:229). This arrangement best ensures that the resulting chimeric adenovirus will maximally express the DNA of interest.

As used herein, the term replication defective adenovirus, refers to a adenovirus that are incapable of self replication within host cells that, absent infection or transfection, do not express at least one adenovirus gene or gene product.

Any number of transcriptional promoters and enhancers may be used in the expression cassette, including, but not limited to, the herpes simplex thymidine kinase promoter, cytomegalovirus promoter/enhancer, SV40 promoters, and retroviral long terminal repeat (LTR) promoter/enhancers. Of special interest are any of a number of well characterized retroviral promoters, particularly the Moloney murine leukemia virus (MLV) LTR promoter and the human immunodeficiency virus (HIV) LTR.

According to one embodiment of the present invention, recombinant DNA techniques have been used to construct expression cassettes in plasmid pXCJ1.1 which comprise genes coding for the murine or human forms of granulocyte macrophage colony stimulating factor (GM-CSF), which have been placed under the transcriptional control of the Moloney murine leukemia virus (MLV) long terminal repeat (LTR). In a further embodiment, an SV40 poly-adenylation sequence flanks the 3' end of the GM-CSF gene. Thus, the transcript produced by either GM-CSF expression cassette is transcribed using the MLV LTR promoter and enhancer sequences, poly-adenylated using an SV40 poly-adenylation sequence, spliced using the MLV splice donor and splice acceptor sequences, and the mRNA is presumably translated using the endogenous MLV translation initiation sequence of the MLV *gag* gene. By engineering the DNA expression cassette such that the resulting transcript surrounds the coding region with naturally occurring viral control sequences, near optimum mRNA stability is obtained. Thus, as used herein, the terms "DNA expression cassette" or simply "expression cassette" both refer to a DNA molecule comprising a eucaryotic promoter and/or enhancer region, a DNA of interest to be transcribed by the promoter, and a substantially noncoding 3' region of DNA that facilitates the stability, polyadenlyation, or splicing of the transcript.

The GM-CSF expression cassette is inserted into a replication defective helper adenovirus via homologous recombination after two circular plasmids (one containing the GM-CSF expression cassette and the other containing the replication defective adenovirus genome) are co-transfected into the appropriate cell line (see Fig. 1). Using this system, only the specifically desired chimeric adenovirus are packaged. The resulting chimeric adenovirus expresses a mammalian gene (human or murine GM-CSF) that is expressed under the transcriptional and translational control of MLV and SV40 control sequences. The chimeric adenovirus can subsequently be purified by any of a number of well established techniques including, but not limited to, plaque

purification, purification by limiting dilution, or the like. Purified chimeric adenovirus can then be propagated to relatively high titers by infection of appropriate host cells, for example 293 cells (human kidney epithelial cells which constitutively produce adenovirus E1A). Although the chimeric adenovirus infections will generally produce highly concentrated viral preparations, one may elect to further concentrate and purify the chimeric adenovirus to achieve titers of about 1-5x10^{11} plaque forming units (pfu)/ml) by CsCl density equilibrium centrifugation (followed by dialysis), ultrafiltration, or the like.

The resulting chimeric adenovirus, designated Ad.mGM-CSF (murine GM-CSF) or Ad.hGM-CSF (human GM-CSF), have been shown to be useful for the production of microgram quantities (as quantified by enzyme linked immunosorbent assay, or ELISA) of GM-CSF in infected NIH 3T3 cells (see Table 1). The properties of Ad.hGM-CSF and Ad.mGM-CSF make both ideally suited for applications where GM-CSF expression by any of a broad range of target cells may be desired.

Of particular interest is the use of Ad.hGM-CSF or Ad.mGM-CSF to transduce primary tumor cells. It has previously been established that vaccinations with tumor cells engineered to secrete GM-CSF can stimulate anti-tumor immunity in mice (Dranoff et al. (1993) Proc. Natl. Acad. Sci. U.S.A. 90:3539-3543. Ad.hGM-CSF has been used to transduce primary human melanoma, renal cell carcinoma, and colon carcinoma cells which subsequently produced microgram quantities (about 1-5μg/10^6 cells) of human GM-CSF (see Tables 2a-d). Additionally, Ad.mGM-CSF has been used to infect and transduce murine B16 melanoma cells which may subsequently be irradiated (using about 5,000 rads) and assessed for efficacy as an anti-melanoma vaccine.

Ad.hGM-CSF was also injected into Balb/c or SCID mice at various anatomical locations, and *in vivo* expression of GM-CSF was detected and quantified by ELISA (see Figs. 5 & 6).

Ad.hGM-CSF has been deposited (received at the ATCC on September 23, 1994) at the American Type Culture Collection,

Rockville, MD, under the accession number _______ under the terms of the Budapest Treaty. Applicants further agree to make this deposit available, without restriction to responsible third parties upon the granting of a patent from this application in the United States, and comply with existing laws and regulations pertaining thereto, without limitation, except as to third parties adherence to applicants rights as prescribed by the claims of a patent issuing from this application.

As described briefly above and in detail in the Examples, the present invention provides a method of producing chimeric adenovirus comprising the recombinatory insertion of a DNA expression cassette contained in a circular plasmid into a replication deficient helper adenovirus genome contained in a circular plasmid to produce a chimeric adenovirus capable of transducing mammalian cells. The use of two circular plasmids is an important feature of the method of the present invention, since there is no need to linearize the adenoviral helper genome prior to cotransfection.

The chimeric adenovirus of the present invention exhibit very high infectivity and thus high levels of cellular transduction and expression of a DNA of interest. In addition to the specifically disclosed GM-CSF genes, modified forms of the GM-CSF genes may be utilized which have been altered by deletion or insertion, or to optimize codon usage for the specific target cells intended. DNA expression cassettes may also be constructed which allow the subsequent production of chimeric adenovirus which are capable of transducing any of a number of heterologous mammalian genes (i.e., DNAs of interest, subject to the restriction that the net size of the insert is less the about 9 kb in length).

Besides GM-CSF, other heterologous genes of particular interest include, but are not limited to, nerve growth factor (NGF), tyrosine hydroxylase (TH), ciliary neurotropic factor (CNTF), brain-derived neurotropic factor (BDNF), factors VIII and IX, tissue plasminogen activator (tPA), interleukins 1-2

and 4-6, tumor necrosis factor-α (TNF-α), α or γ interferons, and erythropoietin. Chimeric adenovirus that express any of the above genes, or portions thereof, may be particularly useful for the treatment of mammalian diseases or disorders related to aberrant or deficient levels of the corresponding polypeptides or proteins in a given individual. Alternatively, chimeric adenovirus containing the genes for these factors may also be used to generate transient expression of the factors in vivo as required to therapeutically treat medical crisis. For instance, an infusion of chimeric adenovirus containing a tPA expression cassette would provide transient expression of tPA during the critical period following a heart-attack or stroke.

The high efficiency transduction inherent in the chimeric adenovirus system makes them particularly well suited for the treatment of genetic or inherited disease, as well as the treatment of acquired disease. For instance, chimeric adenovirus may be used to deliver genes into a variety of cell types to correct genetic defects associated with diseases including but not limited to β-thalassemia, phenylketonuria, sickle-cell anemia, cystic fibrosis, or adenosine deaminase deficiency.

The chimeric adenovirus of the present invention may be used to transduce mammalian cells either in vitro or in vivo. Where transduction in vitro is contemplated, cells may be infected at multiplicities of infection (moi's) of between about 1:1 to about 5000:1, and generally in the range of about 100:1 to about 2,500:1. Moi's of up to about 1000:1 have produced good expression of the DNA of interest without evidence of serious cellular toxicity effects, and moi's of about 200:1 have resulted in no toxicity. Using similar methodologies, chimeric adenovirus may be used to infect resected primary tissue or cells which may subsequently be reintroduced into the body of an individual by established surgical or medical procedures.

Where diagnostic, therapeutic or medicinal use of chimeric adenovirus is contemplated, chimeric adenovirus

capable of transducing and expressing the DNA of interest may be introduced in vivo by any of a number of established methods. For instance, chimeric adenovirus may be administered by inhalation. Alternatively, chimeric adenovirus suspensions may also administered by intravenous (I.V.), intraperitoneal (I.P.), or intramuscular (I.M.) injection.

The chimeric adenovirus may also be injected directly into tumors. To prove the feasibility of this concept, a chimeric adenovirus which encodes a bacterial lacZ gene was injected into B16 melanoma tumors in C57 mice. Following injection, adenovirus mediated transduction and in vivo expression of β-galactosidase was observed in the tumors.

Other in vivo studies have established that a single bolus of as much as about 10^9 pfu (in 100μl total volume) of Ad.hGM-CSF can be injected (I.V. or I.M.) into mice without apparent toxicity effects (see Fig. 4A).

Possible cell types or tissues that may serve as targets for chimeric adenovirus gene delivery include, but are not limited to, hepatocytes, fibroblasts, endothelial cells, bone marrow stem cells, lymphocytes, neural tissue, astrocytes, alveolar tissue, and granulocytes.

An additional embodiment of the present invention is chimeric adenovirus containing expression cassettes which further comprise a specific retroviral Psi-packaging sequence. More particularly, a Psi-packaging sequence which corresponds to that recognized and used by any of a number of ecotropic and amphotropic Moloney murine leukemia virus packaging cell lines including, but not limited to, PA317 or PsiCRIP.

Where the above expression cassette of the chimeric adenovirus further encodes at least a portion of an MLV 3' LTR sequence (minimally comprising the U3 and R regions of the LTR) located distal to the gene of interest, the chimeric adenovirus may be used to transiently infect MLV packaging cell lines and produce amphotropic or ecotropic retrovirus which package RNA genomes transcribed by the expression cassette of the chimeric adenovirus. Infection of the

appropriate cells by the resulting retrovirally packaged chimeric adenovirus transcripts will result in the integration and stable expression of the DNA of interest contained in the expression cassette of the chimeric adenovirus. The chimeric adenovirus described above provide the user with increased versatility relative to previously disclosed retroviral or adenoviral transduction vectors. This is because a single chimeric adenovirus allows the user to choose between the increased storage life, infectivity, and transient expression inherent in the high titer chimeric adenovirus system, or the stable integration and expression inherent in the MLV packaging system. Alternatively, an optimal mixture of the two delivery systems may be preferred. Thus, the present invention also provides for replication defective chimeric adenovirus which contain an expression cassette which further comprises nucleotide sequence corresponding to a MLV Psi-packaging site.

An additional embodiment of the present invention is chimeric adenovirus which place the expression of genes whose products are toxic to the cell under the strict control of a trans-activated promoter, such as an HIV LTR promoter. Toxic genes which may be employed in these vectors include, but are not limited to, sequence coding for diphtheria toxin A chain, polio virus protein 2A, and the like (or modified forms thereof). Since the HIV promoter generally requires virally encoded trans- activators, chimeric adenovirus will generally only express the toxic products (hence killing the cells) in HIV infected cells. Thus, since the expression of genes contained in chimeric adenovirus is not dependent on cell division or proliferation (unlike retrovirally expressed genes), the above chimeric adenovirus may find utility in targeting and killing non-replicating or quiescent HIV-infected cells.

The present invention will now be illustrated by the following examples, which are not intended to be limiting in any way.

6. EXAMPLES

6.1. CONSTRUCTION OF THE PXCJL-GMCSF PLASMID

The starting plasmid, designated PXCJL1, was constructed from a modified Ad5 adenovirus genome cloned into pBR322. A deletion was made from the map units 1.3 to 9.3, and a multiple cloning site was inserted at the unique XbaI site. This construct was obtained from Dr. Frank Graham of McMaster University (McGrory, W.J. et al., Virology 163: 614-617, 1988).

The cDNA for human GM-CSF, along with upstream packaging and splicing sequences and the complete MLV 5' LTR, were isolated from plasmid MFGs-GM-CSF. MFGs is an unpublished three nucleotide modification of the MFG vector, as represented by MFG-GM-CSF (Dranoff, et al., Proc. Natl. Acad. Sci. 90:3539-3543, 1993; the modification has no effect on expression levels or transduction efficiencies). MFGs-GM-CSF DNA was first digested to completion with HindIII and BamHI and the ends were blunt-ended with the Klenow fragment. The plasmid fragments were separated by electrophoresis on a 1% agarose gel, and the 2.7 kb fragment extending from the 5' LTR to the 3' end of the GM-CSF cDNA was purified from the gel (Fragment 1).

The GM-CSF cDNA and associated sequences were then subcloned into the multiple cloning site of PXCJL1 using standard techniques (Sambrook, et al. Molecular Cloning: A Laboratory Manual (1989)). The PXCJL1 plasmid was digested to completion with XbaI, the ends were blunt-ended (end-filled) with Klenow and treated with bacterial alkaline phosphatase. This linearized vector fragment was purified from a 1% agarose cell following electrophoresis (Fragment 2). The purified GM-CSF cDNA (Fragment 1) was blunt-end ligated to the linearized PXCJL1 with T4 ligase to generate the intermediate plasmid PXCJL GM-CSF(I). XbaI and BamHI sites were regenerated in the intermediate plasmid only if the insert was in the correct orientation, as determined by restriction endonuclease (EcoRI and BamHI) analysis.

To insert the SV40 polyadenylation sequence at the 3' end of the GM-CSF cDNA, PXCJL GM-CSF(I) was digested with BamHI and SalI, and the linearized fragment was isolated from a 1% agarose gel following electrophoresis (Fragment 3). The SV40 polyadenylation sequence was generated by polymerase chain reaction (PCR) using the pRC/CMV vector as the DNA template. The PCR primers were designed as follows:

the sense primer containing the BamHI site-

GAG GAT CCT ATC GCC TTC TTG ACG

and the antisense primer containing the SalI site-

GAG TCG ACT AAA CAA GTT GGG GTG.

PCR conditions were 95°C for 1 min., 55°C for 2 min., and 72°for 3 minutes, for 35 cycles. The PCR product was cloned into a TA plasmid and sequenced. The product with the correct SV40 poly(A) sequence was digested with BamHI and SalI and the 216 bp SV40 poly(A) sequence was ligated to PXCJL GM-CSF(I) (Fragment 3) with T4 ligase.

The resulting cDNA expression plasmid, PXCJL, GM-CSF, contains the entire GM-CSF cassette, including the 5' MLV LTR, Psi-packaging and splicing sequences, the GM-CSF cDNA, and the SV40 poly (A) sequences, flanked by adenovirus sequences. Both murine and human GM-CSF cDNA were subcloned into PXCJL1 following the same strategy.

6.2. TRANSFECTION AND ISOLATION OF RECOMBINANT VIRUS

To generate recombinant virus, a replication deficient form of the adenoviral genome in circular form (pJM17) was obtained from Dr. Frank Graham. Techniques for transfection of 293 cells (a human kidney epithelial cell line), overlaying plates with agar-containing medium, picking and analysis of recombinant virus clones were carried out following the methods described by Graham and Prevec ("Manipulation of Adenovirus Vectors", in Gene Transfer and Expression Protocols, E.J. Murray, ed.). Briefly, 293 cells in 100 mm dishes were co-transfected with 10μg of pJM17 and 15μg of PXCJL-GMCSF plasmid by the calcium phosphate method following the standard transfection protocol. 36 hours after

transfection, cells were overlaid with 0.8% Noble agar containing DMEM with 10% heat inactivated fetal calf serum.

Plaques visible by 8 days after transfection were picked and resuspended in 1 ml of medium and freeze-thawed three times to release the virus. These supernatants were used as viral lysates in subsequent experiments. 0.2 ml of the viral supernatant from each individual plaque was added to the 1 ml of medium and used to infect confluent monolayers of 293 cells in a 6-well plate for four hours. After 24 hours, the cells began to show complete cytopathic effects.

At this time the colonies were harvested, and the medium was analyzed for GM-CSF secretion. The cells were lysed by three rounds of freeze-thaw, and the medium was used to infect NIH 3T3 cells in a 6-well plate. 80% confluent monolayers of NIH 3T3 cells in a 6-well plate were infected with 0.1 ml of crude virus stock in 1 ml of medium for four hours. 24 hours after infection fresh growth medium was added, and the GM-CSF secreted for the next 24 hours was analyzed by ELISA. The values for GM-CSF produced by Ad/human GM-CSF and Ad/mouse GM-CSF-transduced NIH 3T3 cells ranged from 300-400ng in 24 hours.

A schematic diagram of the recombination protocol used to generate Ad.hGM-CSF and Ad.mGM-CSF is presented in Figure 1.

6.3. <u>PLAQUE PURIFICATION OF RECOMBINANT VIRUS</u>

Confluent monolayers of 293 cells in 100mm dishes plated on day 1 were infected in 5 ml of medium on day 2 with 0.1 ml of viral supernatant obtained by resuspending virus containing agar block, as described above. After 1 hour of infecting at 37°C, the virus-containing medium was removed and overlaid with the agar-containing medium that had been prepared earlier. The cells were incubated at 37°C for 4-5 days and well isolated plaques were picked and analyzed for the ability to transduce NIH 3T3 cells with GM-CSF, as described earlier.

6.4. PURIFICATION AND AMPLIFICATION OF CHIMERIC ADENOVIRUS

Concentrated virus stocks were prepared from infected 293 cells. Confluent monolayers of 293 cells in 150mm dishes were infected with 5-10pfu/cell and after 36 hours when all the cells began to exhibit complete CPE, the cells were collected and resuspended in 5 ml of 0.1M Tris, pH 8.0. The virus was released from the cell pellets by three freeze-thaw cycles. After sonicating the cell lysate, 1.8 ml of saturated cesium chloride (in 10mM Tris, pH 8.0, 1 mM EDTA) was added to 3.1 ml of the cell lysate. This was centrifuged at 30,000 rpm in a SW 41 rotor for 20 hours. The virus band was collected and repurified by CsCl banding. The purified virus was then dialyzed against 10mM Tris/1 mM $MgCl_2$, pH 7.4, and stored in 10% glycerol at -70°C.

6.5. TRANSDUCTION OF NIH 3T3 CELLS WITH Ad.hGM-CSF AND Ad.mGM-CSF

NIH 3T3 cells were infected with purified virus at different multiplicities of infection (moi) for four hours, supernatants from 24-48 hours post-infection were collected and GM-CSF secretion was measured by ELISA. Results are shown in Table 1.

Table 1. Expression of human GM-CSF ($\mu g/1x10^6$ cells/24 hr) in 3T3 cells.

TABLE 1.

moi	500	250	100	50
Ad.hGM-CSF	2.1	1.4	0.41	0.125
Ad.mGM-CSF	1.6	0.9	0.375	0.08

6.6. TRANSDUCTION OF PRIMARY HUMAN TUMOR CELLS WITH Ad.hGM-CSF Virus

Primary cultures of human melanoma, renal cell carcinoma, colon carcinoma and colorectal tumor cells were established and were transduced with Ad.hGM-CSF virus. The cultures were infected with Ad.hGM-CSF at different moi's for 4-8 hours,

supernatants were collected at 24-48 hours post-infection, and GM-CSF secretion was measured by ELISA. Results for the various cell types are presented in Tables 2a-d.

Tables 2a-d. Expression of GM-CSF ($\mu g/1x10^6$ cells/24 hour) in Ad.hGM-CSF transduced primary tumor cells.

TABLE 2a.

moi	5000	1000	500	250	125	62.5	50
Melanoma-1 (P2)	2.3	12.6	5.4				1.1
Melanoma-2 (P2)		9.4	3.2	1.8	0.93	0.47	
Melanoma-3 (P2)		2.4	2.4	0.09	0.09	0.045	

TABLE 2b.

moi	5000	2500	1000	500	100
Renal Cell carcinoma (P3)	4.1	6.7	7.5	4.7	2.1

TABLE 2c.

moi	1000	200	100	20	10
Colorectal cells (P1)	0.15	1.8	1.5	0.42	0.22

TABLE 2d.

moi	5000	1000	500	50
Colon carcinoma (P1)	13.8	23.6	6.7	0.9

By comparison, transduction of the same types of human tumor cells by recombinant retrovirus expressing human GM-CSF results in expression in the range of 40-500 ng/$1x10^6$ cells/24 hours.

6.7. DELIVERY OF HUMAN GM-CSF INTO BALB/C MICE

To test for the ability of Ad.hGM-CSF to transduce mammalian cells *in vivo*, one month old Balb/C mice were injected intramuscularly (thigh muscle) with 100μl of purified virus at a concentration of either 10^{10} or 10^{9} pfu/ml. Transient expression of human GM-CSF was quantified by ELISA of serum samples taken from the mice at 2, 5, 7, 9, 14, and 21 days post infection. The data are presented in Figures 4A and 4B. Mice injected with 10^{9} pfu (Fig. 4A) exhibited peak expression of human GM-CSF five days after injection with transient expression tapering down to undetectable levels between seven to nine days after injection. Mice injected with 10^{8} pfu (Fig. 4B) also showed peak expression at about five days post injection but continued to express human GM-CSF until between nine to fourteen days after injection. These data clearly indicate that Ad.hGM-CSF transduces cells *in vivo*, and further mediates transient expression of human GM-CSF.

6.8. REPEATED INJECTION OF Ad.hGM-CSF INTO ADULT BALB/C MICE

To test whether Ad.hGM-CSF could also mediate transient expression of human GM-CSF in adult mice, and whether or not the route of injection substantially affected expression, four month old Balb/C mice were injected with 10^{8} pfu of Ad.hGM-CSF either intravenously (I.V.) or intramuscularly (I.M.). Serum samples were drawn at 3, 7, 14, and 31 days after injection and assayed for GM-CSF levels by ELISA. Serum levels of GM-CSF were generally lower than those observed in one month old mice, peaked between three to seven days after injection, and were undetectable fourteen days after infection.

Thirty one days after the initial injection the mice were reinjected (I.M.) with 10^{9} pfu of Ad.hGM-CSF and serum samples were drawn and analyzed for GM-CSF at 2, 4, and 9 days after reinjection. After reinjection, serum levels of GM-CSF peaked after two days and were undetectable after four days. The mode of primary injection apparently made little difference (see Fig. 5).

6.9. REPEATED INJECTION OF Ad.hGM-CSF INTO SCID MICE

To test whether an immune response might be the cause of the reduced expression of GM-CSF after reinjection, experiment 6.8 was essentially repeated using SCID (severe combined immunodeficiency) mice with the exception that Ad.hGM-CSF were only administered I.V.. As can be seen in Figure 6, SCID mice continued to express GM-CSF up to twenty eight days after initial infection and forty three days after I.M. reinjection of 10^9 pfu of Ad.hGM-CSF. These data (presented in Fig. 6) indicate that the diminution of GM-CSF levels in adult Balb/C mice seen in experiment 6.8 may be due to immune reaction to the adenovirus antigens expressed by the replication deficient genome of Ad.hGM-CSF.

All publications and patents mentioned in the above specification are herein incorporated by reference. The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the virus deposited since the deposited embodiment is intended as a simple illustration of one aspect of the invention and any virus that are functionally equivalent are within the scope of this invention. Various modifications of the invention in addition to those specifically shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Srinivas, Shankara
Dwarki, Varavani
Nijjar, Tarlochan

(ii) TITLE OF INVENTION: Chimeric Adenovirus for Gene Delivery

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Pennie & Edmonds
(B) STREET: 2730 Sand Hill Road
(C) CITY: Menlo Park
(D) STATE: California
(E) COUNTRY: U.S.A.
(F) ZIP: 94025

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US To be assigned.
(B) FILING DATE: 22-SEP-1994
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Halluin, Albert P.
(B) REGISTRATION NUMBER: 25,227
(C) REFERENCE/DOCKET NUMBER: 8141-119-999

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 415-854-3660
(B) TELEFAX: 415-854-3694
(C) TELEX: 66141 PENNIE

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9629 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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What is claimed is:

1. A chimeric adenovirus which comprises:

a replication deficient adenovirus genome; and

a DNA expression cassette comprising:

a eucaryotic promoter and/or enhancer region; nucleotide sequence corresponding to a MLV Psi-packaging site; a DNA of interest to be transcribed by said promoter; and a substantially noncoding 3' DNA which facilitates the stability, polyadenlyation, or splicing of the transcript.

2. The chimeric adenovirus of Claim 1 wherein said DNA of interest is drawn from the group comprising:

granulocyte macrophage colony stimulating factor (GM-CSF); nerve growth factor (NGF); tyrosine hydroxylase (TH); ciliary neurotropic factor (CNTF); brain-derived neurotropic factor (BDNF); factors VIII and IX; tissue plasminogen activator (tPA); interleukins 1-2 and 4-6; tumor necrosis factor-α (TNF-α); α or γ interferons; or erythropoietin.

3. The chimeric adenovirus of Claim 1 wherein said DNA of interest is the gene encoding human granulocyte macrophage colony stimulating factor.

4. The chimeric adenovirus of Claim 1 wherein said DNA of interest is the gene encoding murine granulocyte macrophage colony stimulating factor.

5. A chimeric adenovirus which comprises:

a replication deficient adenovirus genome; and

a DNA expression cassette consisting essentially of an MLV LTR promoter and enhancer region; nucleotide sequence corresponding to a MLV Psi-packaging site; a gene encoding human granulocyte macrophage colony stimulating factor; and an SV40 polyadenylation sequence.

6. A chimeric adenovirus which comprises:

a replication deficient adenovirus genome; and

a DNA expression cassette consisting essentially of an MLV LTR promoter and enhancer region; nucleotide sequence corresponding to a MLV Psi-packaging site; a gene encoding murine granulocyte macrophage colony stimulating factor; and an SV40 polyadenylation sequence.

7. The use of the chimeric adenovirus of Claim 1 in the treatment of mammalian disease and disorders.

8. The use of the chimeric adenovirus of Claim 2 to transduce mammalian cells.

9. The use of the chimeric adenovirus of Claim 3 to transduce tumor cells.

10. The use of the chimeric adenovirus of Claim 4 to transduce tumor cells for use as anti-tumor vaccines.

11. A method of producing chimeric adenovirus comprising:

the recombinatory insertion of a DNA expression cassette into a replication deficient helper adenovirus genome contained in a circular plasmid to produce a chimeric adenovirus capable of transducing mammalian cells.

12. The method of Claim 11 wherein said DNA expression cassette comprises:

a eucaryotic promoter and/or enhancer region;

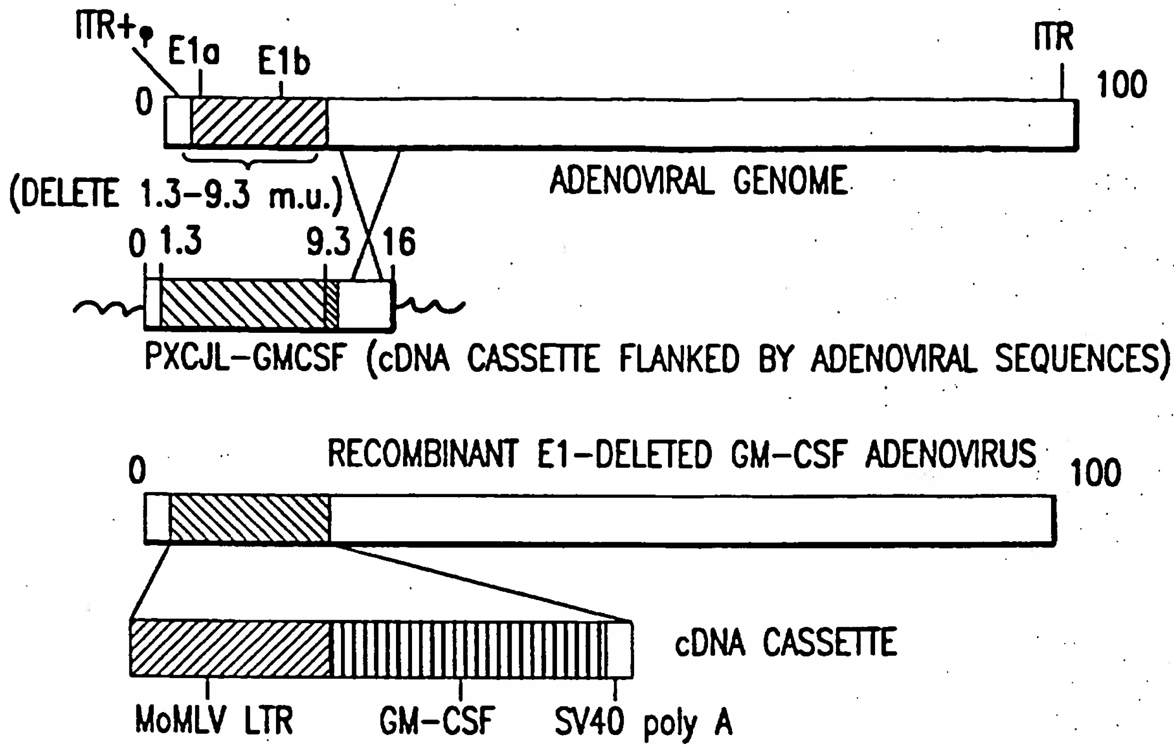
a DNA of interest to be transcribed by said promoter; and

a 3' substantially noncoding DNA that facilitates the stability, polyadenlyation, or splicing of the transcript.

13. The method of Claim 12 wherein said DNA of interest is drawn from the group comprising:

granulocyte macrophage colony stimulating factor (GM-CSF); nerve growth factor (NGF); tyrosine hydroxylase (TH); ciliary neurotropic factor (CNTF); brain-derived neurotropic factor (BDNF); factors VIII and IX; tissue plasminogen activator (tPA); interleukins 1-2 and 4-6; tumor necrosis factor-α (TNF-α); α or γ interferons; or erythropoietin.

14. The method of Claim 12 wherein said DNA of interest is the gene encoding granulocyte macrophage colony stimulating factor.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E1a
E1b
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9.3
16
PXCJL–GMCSF (cDNA CASSETTE FLANKED BY ADENOVIRAL SEQUENCES)
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0
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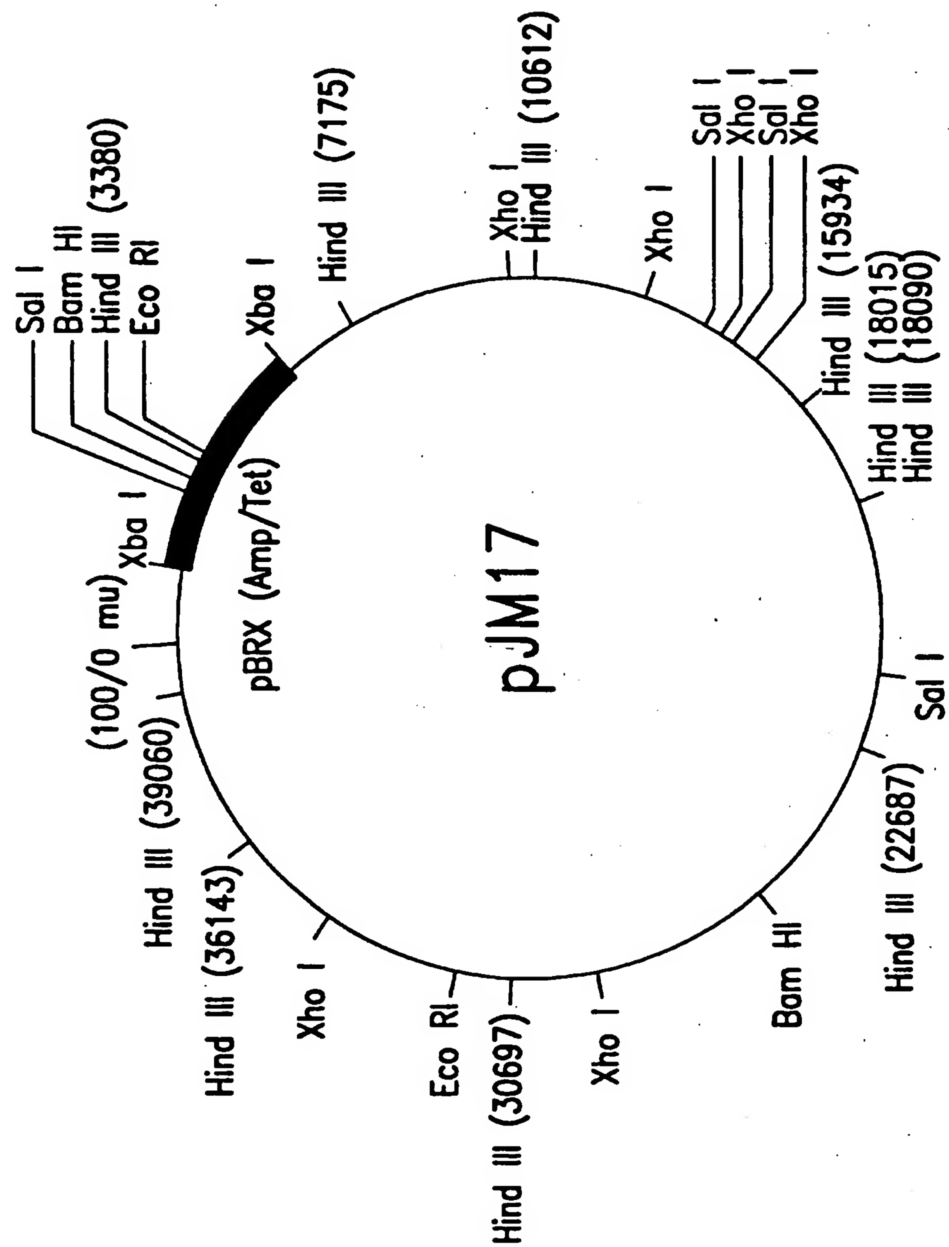
cDNA CASSETTE
MoMLV LTR
GM–CSF
SV40 poly A

FIG.1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pBRX (Amp/Tet)
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Eco RI
Xba I
Hind III (7175)
Xho I
Hind III (10612)
Xho I
Sal I
Xho I
Sal I
Xho I
Hind III (15934)
Hind III (18015)
Hind III (18090)
Sal I
Hind III (22687)
Bam HI
Xho I
Hind III (30697)
Eco RI
Xho I
Hind III (36143)
Hind III (39060)
(100/0 mu)
Xba I

FIG.2

GAATTCCATC ATCAATAATA TACCTTATTT TGGATTGAAG CCAATATGAT AATGAGGGGG 60
TGGAGTTTGT GACGTGGCGC GGGGCGTGGG AACGGGGCGG GTGACGTAGT AGTGTGGCGG 120
AAGTGTGATG TTGCAAGTGT GGCGGAACAC ATGTAAGCGA CGGATGTGGC AAAAGTGACG 180
TTTTTGGTGT GCGCCGGTGT ACACAGGAAG TGACAATTTT CGCGCGGTTT TAGGCGGATG 240
TTGTAGTAAA TTTGGGCGTA ACCGAGTAAG ATTTGGCCAT TTTCGCGGGA AAACTGAATA 300
AGAGGAAGTG AAATCTGAAT AATTTTGTGT TACTCATAGC GCGTAATATT TGTCTAGGGC 360
CGCGGGGACT TTGACCGTTT ACGTGGAGAC TCGCCCAGGT GTTTTTCTCA GGTGTTTTCC 420
GCGTTCCGGG TCAAAGTTGG CGTTTTATTA TTATAGTCTC TAGAGCTTTG CTCTTAGGAG 480
TTTCCTAATA CATCCCAAAC TCAAATATAT AAAGCATTTG ACTTGTTCTA TGCCCTAGGG 540
GGCGGGGGGA AGCTAAGCCA GCTTTTTTTA ACATTTAAAA TGTTAATTCC ATTTTAAATG 600
CACAGATGTT TTTATTTCAT AAGGGTTTCA ATGTGCATGA ATGCTGCAAT ATTCCTGTTA 660
CCAAAGCTAG TATAAATAAA AATAGATAAA CGTGGAAATT ACTTAGAGTT TCTGTCATTA 720
ACGTTTCCTT CCTCAGTTGA CAACATAAAT GCGCTGCTGA GCAAGCCAGT TTGCATCTGT 780
CAGGATCAAT TTCCCATTAT GCCAGTCATA TTAATTACTA GTCAATTAGT TGATTTTTAT 840
TTTTGACATA TACATGTGAA TGAAAGACCC CACCTGTAGG TTTGGCAAGC TAGCTTAAGT 900
AACGCCATTT TGCAAGGCAT GGAAAAATAC ATAACTGAGA ATAGAAAAGT TCAGATCAAG 960
GTCAGGAACA GATGGAACAG CTGAATATGG GCCAAACAGG ATATCTGTGG TAAGCAGTTC 1020
CTGCCCCGGC TCAGGGCCAA GAACAGATGG AACAGCTGAA TATGGGCCAA ACAGGATATC 1080
TGTGGTAAGC AGTTCCTGCC CCGGCTCAGG GCCAAGAACA GATGGTCCCC AGATGCGGTC 1140
CAGCCCTCAG CAGTTTCTAG AGAACCATCA GATGTTTCCA GGGTGCCCCA AGGACCTGAA 1200
ATGACCCTGT GCCTTATTTG AACTAACCAA TCAGTTCGCT TCTCGCTTCT GTTCGCGCGC 1260
TTCTGCTCCC CGAGCTCAAT AAAAGAGCCC ACAACCCCTC ACTCGGGGCG CCAGTCCTCC 1320
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CTTGTGGTCT CGCTGTTCCT TGGGAGGGTC TCCTCTGAGT GATTGACTAC CCGTCAGCGG 1440
GGGTCTTTCA TTTGGGGGCT CGTCCGGGAT CGGGAGACCC CTGCCCAGGG ACCACCGACC 1500
CACCACCGGG AGGTAAGCTG GCCAGCAACT TATCTGTGTC TGTCCGATTG TCTAGTGTCT 1560
ATGACTGATT TTATGCGCCT GCGTCGGTAC TAGTTAGCTA ACTAGCTCTG TATCTGGCGG 1620
ACCCGTGGTG GAACTGACGA GTTCGGAACA CCCGGCCGCA ACCCTGGGAG ACGTCCCAGG 1680
GACTTCGGGG GCCGTTTTTG TGGCCCGACC TGAGTCCTAA AATCCCGATC GTTTAGGACT 1740

FIG.3A

CTTTGGTGCA CCCCCCTTAG AGGAGGGATA TGTGGTTCTG GTAGGAGACG AGAACCTAAA 1800
ACAGTTCCCG CCTCCGTCTG AATTTTTGCT TTCGGTTTGG GACCGAAGCC GCGCCGCGCG 1860
TCTTGTCTGC TGCAGCATCG TTCTGTGTTG TCTCTGTCTG ACTGTGTTTC TGTATTTGTC 1920
TGAAAATATG GGCCCGGGCT AGACTGTTAC CACTCCCTTA AGTTTGACCT TAGGTCACTG 1980
GAAAGATGTC GAGCGGATCG CTCACAACCA GTCGGTAGAT GTCAAGAAGA GACGTTGGGT 2040
TACCTTCTGC TCTGCAGAAT GGCCAACCTT TAACGTCGGA TGGCCGCGAG ACGGCACCTT 2100
TAACCGAGAC CTCATCACCC AGGTTAAGAT CAAGGTCTTT TCACCTGGCC CGCATGGACA 2160
CCCAGACCAG GTCCCCTACA TCGTGACCTG GGAAGCCTTG GCTTTTGACC CCCCTCCCTG 2220
GGTCAAGCCC TTTGTACACC CTAAGCCTCC GCCTCCTCTT CCTCCATCCG CCCCGTCTCT 2280
CCCCCTTGAA CCTCCTCGTT CGACCCCGCC TCGATCCTCC CTTTATCCAG CCCTCACTCC 2340
TTCTCTAGGC GCCCCCATAT GGCCATATGA GATCTTATAT GGGGCACCCC CGCCCCTTGT 2400
AAACTTCCCT GACCCTGACA TGACAAGAGT TACTAACAGC CCCTCTCTCC AAGCTCACTT 2460
ACAGGCTCTC TACTTAGTCC AGCACGAAGT CTGGAGACCT CTGGCGGCAG CCTACCAAGA 2520
ACAACTGGAC CGACCGGTGG TACCTCACCC TTACCGAGTC GGCGACACAG TGTGGGTCCG 2580
CCGACACCAG ACTAAGAACC TAGAACCTCG CTGGAAAGGA CCTTACACAG TCCTGCTGAC 2640
CACCCCCACC GCCCTCAAAG TAGACGGCAT CGCAGCTTGG ATACACGCCG CCCACGTGAA 2700
GGCTGCCGAC CCCGGGGGTG GACCATCCTC TAGACTGCCA TGTGGCTGCA GAGCCTGCTG 2760
CTCTTGGGCA CTGTGGCCTG CAGCATCTCT GCACCCGCCC GCTCGCCCAG CCCCAGCACG 2820
CAGCCCTGGG AGCATGTGAA TGCCATCCAG GAGGCCCGGC GTCTCCTGAA CCTGAGTAGA 2880
GACACTGCTG CTGAGATGAA TGAAACAGTA GAAGTCATCT CAGAAATGTT TGACCTCCAG 2940
GAGCCGACCT GCCTACAGAC CCGCCTGGAG CTGTACAAGC AGGGCCTGCG GGGCAGCCTC 3000
ACCAAGCTCA AGGGCCCCTT GACCATGATG GCCAGCCACT ACAAGCAGCA CTGCCCTCCA 3060
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GCCTTCTTGA CGAGTTCTTC TGAGCGGGAC TCTGGGGTTC GAAATGACCG ACCAAGCGAC 3300
GCCCAACCTG CCATCACGAG ATTTCGATTC CACCGCCGCC TTCTATGAAA GGTTGGGCTT 3360
CGGAATCGTT TTCCGGGACG CCGGCTGGAT GATCCTCCAG CGCGGGGATC TCATGCTGGA 3420
GTTCTTCGCC CACCCCAACT TGTTTAGTCG ACATCGATAG ATCTGGAAGG TGCTGAGGTA 3480

FIG.3B

CGATGAGACC CGCACCAGGT GCAGACCCTG CGAGTGTGGC GGTAAACATA TTAGGAACCA 3540
GCCTGTGATG CTGGATGTGA CCGAGGAGCT GAGGCCCGAT CACTTGGTGC TGGCCTGCAC 3600
CCGCGCTGAG TTTGGCTCTA GCGATGAAGA TACAGATTGA GGTACTGAAA TGTGTGGGCG 3660
TGGCTTAAGG GTGGGAAAGA ATATATAAGG TGGGGGTCTT ATGTAGTTTT GTATCTGTTT 3720
TGCAGCAGCC GCCGCCGCCA TGAGCACCAA CTCGTTTGAT GGAAGCATTG TGAGCTCATA 3780
TTTGACAACG CGCATGCCCC CATGGGCCGG GGTGCGTCAG AATGTGATGG GCTCCAGCAT 3840
TGATGGTCGC CCCGTCCTGC CCGCAAACTC TACTACCTTG ACCTACGAGA CCGTGTCTGG 3900
AACGCCGTTG GAGACTGCAG CCTCCGCCGC CGCTTCAGCC GCTGCAGCCA CCGCCCGCGG 3960
GATTGTGACT GACTTTGCTT TCCTGAGCCC GCTTGCAAGC AGTGCAGCTT CCCGTTCATC 4020
CGCCCGCGAT GACAAGTTGA CGGCTCTTTT GGCACAATTG GATTCTTTGA CCCGGGAACT 4080
TAATGTCGTT TCTCAGCAGC TGTTGGATCT GCGCCAGCAG GTTTCTGCCC TGAAGGCTTC 4140
CTCCCCTCCC AATGCGGTTT AAAACATAAA TAAAAAACCA GACTCTGTTT GGATTTGGAT 4200
CAAGCAAGTG TCTTGCTGTC TTTATTTAGG GGTTTTGCGC GCGCGGTAGG CCCGGGACCA 4260
GCGGTCTCGG TCGTTGAGGG TCCTGTGTAT TTTTTCCAGG ACGTGGTAAA GGTGACTCTG 4320
GATGTTCAGA TACATGGGCA TAAGCCCGTC TCTGGGGTGG AGGTAGCACC ACTGCAGAGC 4380
TTCATGCTGC GGGGTGGTGT TGTAGATGAT CCAGTCGTAG CAGGAGCGCT GGGCGTGGTG 4440
CCTAAAAATG TCTTTCAGTA GCAAGCTGAT TGCCAGGGGC AGGCCCTTGG TGTAAGTGTT 4500
TACAAAGCGG TTAAGCTGGG ATGGGTGCAT ACGTGGGGAT ATGAGATGCA TCTTGGACTG 4560
TATTTTTAGG TTGGCTATGT TCCCAGCCAT ATCCCTCCGG GGATTCATGT TGTGCAGAAC 4620
CACCAGCACA GTGTATCCGG TGCACTTGGG AAATTTGTCA TGTAGCTTAG AAGGAAATGC 4680
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CATTTCCCAC GCTTTGAGTT CAGATGGGGG GATCATGTCT ACCTGCGGGG CGATGAAGAA 4980
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AGAGCTGCAG CTGCCGTCAT CCCTGAGCAG GGGGGCCACT TCGTTAAGCA TGTCCCTGAC 5160
TCGCATGTTT TCCCTGACCA AATCCGCCAG AAGGCGCTCG CCGCCCAGCG ATAGCAGTTC 5220

FIG.3C

TTGCAAGGAA GCAAAGTTTT TCAACGGTTT GAGACCGTCC GCCGTAGGCA TGCTTTTGAG 5280
CGTTTGACCA AGCAGTTCCA GGCGGTCCCA CAGCTCGGTC ACCTGCTCTA CGGCATCTCG 5340
ATCCAGCATA TCTCCTCGTT TCGCGGGTTG GGGCGGCTTT CGCTGTACGG CAGTAGTCGG 5400
TGCTCGTCCA GACGGGCCAG GGTCATGTCT TTCCACGGGC GCAGGGTCCT CGTCAGCGTA 5460
GTCTGGGTCA CGGTGAAGGG GTGCGCTCCG GGCTGCGCGC TGGCCAGGGT GCGCTTGAGG 5520
CTGGTCCTGC TGGTGCTGAA GCGCTGCCGG TCTTCGCCCT GCGCGTCGGC CAGGTAGCAT 5580
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TTGGAGGAGG CGCCGCACGA GGGGCAGTGC AGACTTTTGA GGGCGTAGAG CTTGGGCGCG 5700
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CCTTCAACCC AGTCAGCTCC TTCCGGTGGG CGCGGGGCAT GACTATCGTC GCCGCACTTA 6000
TGACTGTCTT CTTTATCATG CAACTCGTAG GACAGGTGCC GGCAGCGCTC TGGGTCATTT 6060
TCGGCGAGGA CCGCTTTCGC TGGAGCGCGA CGATGATCGG CCTGTCGCTT GCGGTATTCG 6120
GAATCTTGCA CGCCCTCGCT CAAGCCTTCG TCACTGGTCC CGCCACCAAA CGTTTCGGCG 6180
AGAAGCAGGC CATTATCGCC GGCATGGCGG CCGACGCGCT GGGCTACGTC TTGCTGGCGT 6240
TCGCGACGCG AGGCTGGATG GCCTTCCCCA TTATGATTCT TCTCGCTTCC GGCGGCATCG 6300
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GGTGCGCATG ATCGTGCTCC TGTCGTTGAG GACCCGGCTA GGCTGGCGGG GTTGCCTTAC 6780
TGGTTAGCAG AATGAATCAC CGATACGCGA GCGAACGTGA AGCGACTGCT GCTGCAAAAC 6840
GTCTGCGACC TGAGCAACAA CATGAATGGT CTTCGGTTTC CGTGTTTCGT AAAGTCTGGA 6900
AACGCGGAAG TCAGCGCCCT GCACCATTAT GTTCCGGATC TGCATCGCAG GATGCTGCTG 6960

FIG.3D

GCTACCCTGT GGAACACCTA CATCTGTATT AACGAAGCGC TGGCATTGAC CCTGAGTGAT 7020
TTTTCTCTGG TCCCGCCGCA TCCATACCGC CAGTTGTTTA CCCTCACAAC GTTCCAGTAA 7080
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TGGTCATGAG ATTATCAAAA AGGATCTTCA CCTAGATCCT TTTAAATTAA AAATGAAGTT 8520
TTAAATCAAT CTAAAGTATA TATGAGTAAA CTTGGTCTGA CAGTTACCAA TGCTTAATCA 8580
GTGAGGCACC TATCTCAGCG ATCTGTCTAT TTCGTTCATC CATAGTTGCC TGACTCCCCG 8640
TCGTGTAGAT AACTACGATA CGGGAGGGCT TACCATCTGG CCCCAGTGCT GCAATGATAC 8700

FIG.3E

CGCGAGACCC ACGCTCACCG GCTCCAGATT TATCAGCAAT AAACCAGCCA GCCGGAAGGG 8760
CCGAGCGCAG AAGTGGTCCT GCAACTTTAT CCGCCTCCAT CCAGTCTATT AATTGTTGCC 8820
GGGAAGCTAG AGTAAGTAGT TCGCCAGTTA ATAGTTTGCG CAACGTTGTT GCCATTGCTG 8880
CAGGCATCGT GGTGTCACGC TCGTCGTTTG GTATGGCTTC ATTCAGCTCC GGTTCCCAAC 8940
GATCAAGGCG AGTTACATGA TCCCCCATGT TGTGCAAAAA AGCGGTTAGC TCCTTCGGTC 9000
CTCCGATCGT TGTCAGAAGT AAGTTGGCCG CAGTGTTATC ACTCATGGTT ATGGCAGCAC 9060
TGCATAATTC TCTTACTGTC ATGCCATCCG TAAGATGCTT TTCTGTGACT GGTGAGTACT 9120
CAACCAAGTC ATTCTGAGAA TAGTGTATGC GGCGACCGAG TTGCTCTTGC CCGGCGTCAA 9180
CACGGGATAA TACCGCGCCA CATAGCAGAA CTTTAAAAGT GCTCATCATT GGAAAACGTT 9240
CTTCGGGGCG AAAACTCTCA AGGATCTTAC CGCTGTTGAG ATCCAGTTCG ATGTAACCCA 9300
CTCGTGCACC CAACTGATCT TCAGCATCTT TTACTTTCAC CAGCGTTTCT GGGTGAGCAA 9360
AAACAGGAAG GCAAAATGCC GCAAAAAAGG GAATAAGGGC GACACGGAAA TGTTGAATAC 9420
TCATACTCTT CCTTTTTCAA TATTATTGAA GCATTTATCA GGGTTATTGT CTCATGAGCG 9480
GATACATATT TGAATGTATT TAGAAAAATA AACAAATAGG GGTTCCGCGC ACATTTCCCC 9540
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FIG.3F

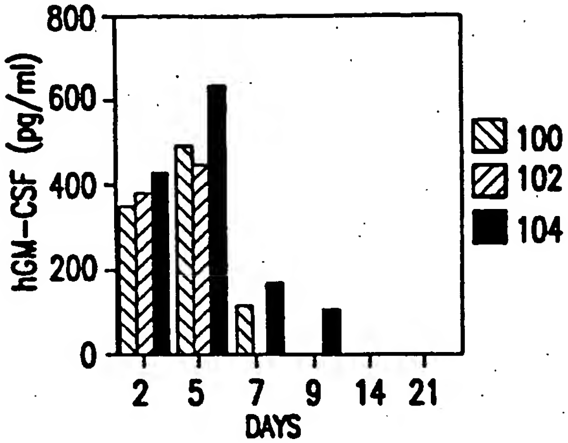

FIG.4A

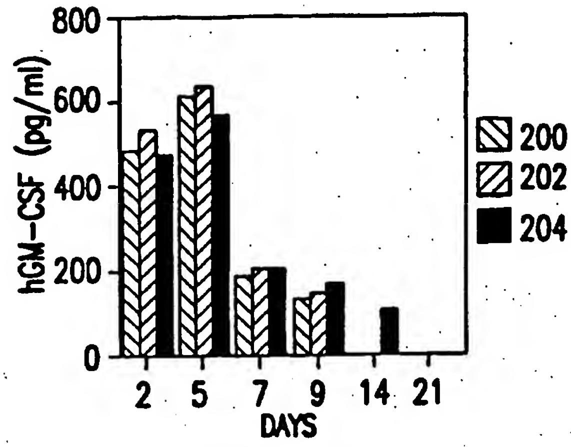

FIG.4B

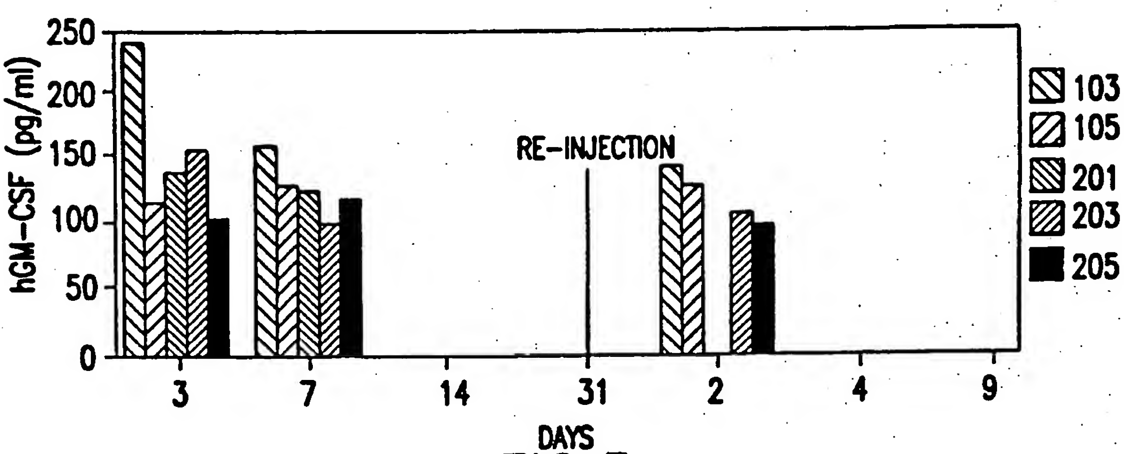

FIG.5

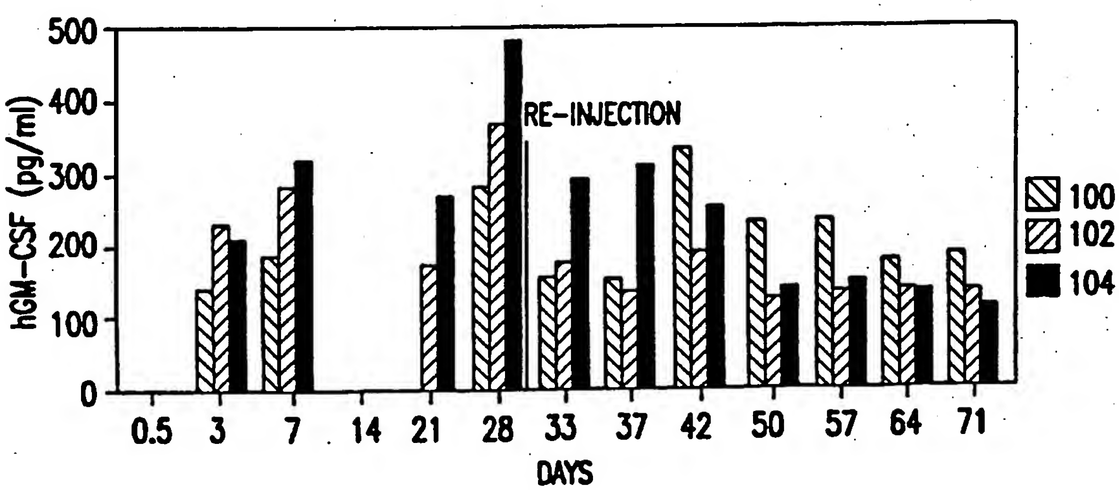

FIG.6

PCT

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(71) Applicant: SOMATIX THERAPY CORPORATION [US/US]; Suite 100, 950 Marina Village Parkway, Alameda, CA 94501 (US).

(72) Inventors: SHANKARA, Srinivas; Apartment E, 2255 San Jose Avenue, Alameda, CA 94501 (US). DWARKI, Varavani; Apartment N, 1175 Broadway Street, Alameda, CA 94501 (US). NIJJAR, Tarlochan; 946 Foxfire Drive, Manteca, CA 95336 (US).

(74) Agents: HALLUIN, Albert, P. et al.; Pennie & Edmonds, 1155 Avenue of the Americas, New York, NY 10036 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

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With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

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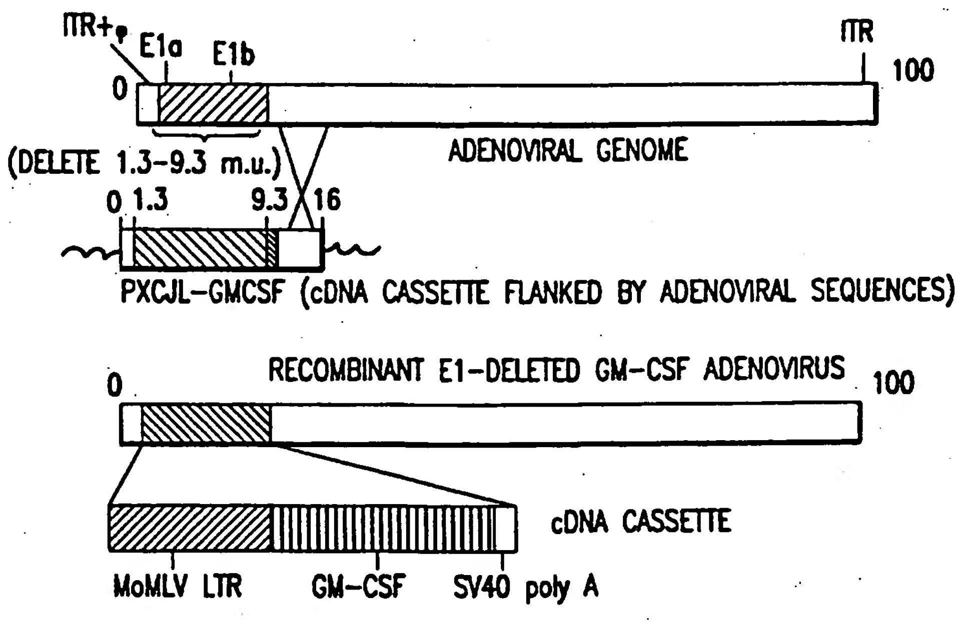
(54) Title: CHIMERIC ADENOVIRUS FOR GENE DELIVERY



(57) Abstract

Chimeric adenovirus capable of transducing mammalian cells with DNA of interest are disclosed. The chimeric adenovirus are useful for the delivery of cloned genes into an individual and are therefore also useful for treating mammalian genetic diseases and disorders.

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GA	Gabon				

INTERNATIONAL SEARCH REPORT

Inter; nal Application No
PCT/US 95/11537

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C07K14/535

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,93 03163 (FONDATION NATIONALE DE TRANSFUSION SANGUINE) 18 February 1993 see page 4, line 19 - page 8, line 18; example 5 ---	1-14
A	EUROPEAN JOURNAL OF NEUROSCIENCE, vol. 5 , no. 10 , 1 October 1993, pages 1287-1291, XP002002600 C.CAILLAUD ET AL.: "Adenoviral vector as a gene delivery system into cultured rat neuronal and glial cells" ---	1-14

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
9 May 1996	23.05.96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Cupido, M

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, 12 April 1994, WASHINGTON US, pages 3054-3057, XP002002601 S-H CHEN ET AL.: "Gene therapy for brain tumors:Regression of experimental gliomas by adenovirus-mediated gene transfer in vivo." see paragraph bridging left and right columns on page 3054 -----	1-14

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11537

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 7,10
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 7 and 10 are directed to a method of treatment of the human body the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 95/11537

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9303163	18-02-93	EP-A-	0596881	18-05-94
		JP-T-	6508982	13-10-94